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The Combinatorial Extension Method Reveals a Sphingolipid Binding Domain on Pancreatic Bile Salt-Dependent Lipase: Role in Secretion

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Summary

Structure similarity searches using a combinatorial extension appoach revealed that a protein fold structurally related to the sphingolipid binding domain (SBD) of HIV-1 gp120 (V3 loop) is present on pancreatic bile salt-dependent lipase (BSDL). A synthetic peptide derived from the predicted V3-like domain of BSDL interacted with reconstituted monolayers of sphingolipids such as GalCer and GlcCer. Using Chinese hamster ovary cells stably transfected with the cDNA encoding the rat BSDL (CHO-3B clone) or pancreatic SOJ-6 cells expressing the human BSDL as models, we showed that the enzyme cofractionates with caveolin-1. The secretion of BSDL by CHO-3B cells was inhibited by permeable drugs affecting rafts structure (D609, PDMP, and filipin). Data suggest that the functional interaction between the BSDL SBD and lipid rafts is physiologically relevant and could be essential for sensing the BSDL folding prior to secretion. A tentative model accounting for the phosphorylation-induced dissociation of BSDL from rafts is presented.

Introduction

Bile salt-dependent lipase (BSDL, E.C.3.1.1.13) is an enzyme involved in the duodenal hydrolysis of cholesteryl esters. The enzyme is synthesized in the endoplasmic reticulum (ER) of pancreatic acinar cells, and then follows the secretory pathway of these cells to be secreted as a component of the pancreatic juice (Lombardo, 2001). BSDL is associated with membranes of the ER and the Golgi apparatus (Bruneau et al., 1995). This association involves a membrane folding multiprotein complex (Bruneau and Lombardo, 1995) in which the Grp94 chaperone helps in the last folding steps (Nganga et al., 2000) of BSDL. The association of BSDL with intracellular membranes could be essential for the complete *O*-glycosylation of a BSDL domain, rich in proline, threonine, serine, and glutamic acid, and referred to as PEST sequences which are signal for rapid degradation of proteins (Rechsteiner and Rogers, 1996). This domain is located within the C-terminal tandemly repeated identical mucin-like sequences of the protein (Reue et al., 1991). We have also demonstrated that the *O*-glycosylation of the mucin-like repeated sequences masks the PEST domain and is required for BSDL secretion (Bruneau et al., 1997). BSDL is released from intracellular membranes in the *trans*-Golgi network (Pasqualini et al., 2000) once phosphorylated at residue Thr340 (Vérine et al., 2001; Pasqualini et al., 1997); then soluble BSDL precipitates with other pancreatic enzymes in condensing vesicles and finally follows the secretion pancreatic pathway until the duodenal lumen where the enzyme accomplishes its physiological role (Lombardo, 2001). We have further shown that BSDL that cannot be correctly folded by Grp94 upon dissociation from the BSDL-Grp94 complex with geldanamycin was directed to the degradation pathway implicating the proteasome (Nganga et al., 2000) and ubiquitination (Le Petit-Thevenin et al., 2001b). Polyunsaturated fatty acid incorporation into lipid membranes of pancreatic cells, lowers the BSDL secretion rate, and increases both retention in microsomes and degradation of the enzyme (Le Petit-Thevenin et al., 2001a). Furthermore, a fraction of BSDL has been detected in the detergent insoluble fractions of human and rat pancreatic microsomes (Bruneau et al., 1995; Bruneau and Lombardo, 1995) which contain raft-associated membrane proteins such as glycosylphosphatidyl inositol (GPI)-anchored proteins (Hooper and Bashir, 1991). All this suggests that BSDL could interact with lipid rafts microdomains during its route toward secretion.

Rafts, also referred to as detergent-insoluble complexes, are membrane microdomains enriched in sphingolipids and cholesterol that are thought to originate from the Golgi apparatus. Rafts have been proposed to function as a sorting platform for the apical delivery of plasma membrane proteins (Simons and Ikonen, 1997; Wang et al., 2002), to play a role in signal transduction (Horejsi et al., 1999; Langlet et al., 2000), infection (Hug et al., 2000; Lafont, et al., 2002), and secretion (Martin-Belmonte et al., 2000). Raft association is mediated either by the transmembrane domain (Lin et al., 1998) or by the GPI anchor (Harder and Simons, 1997) of proteins. However, in a recent study Mahfoud et al. (2002a) have identified a common sphingolipid binding domain in Alzheimer β-amyloid peptide, prion protein, and gp120 HIV-1 protein. The characterization of this common sphingolipid binding domain or SRB (Fantini, 2003) on these proteins underscores the role of membrane rafts in pathogenesis (Fantini et al., 2002) as HIV exploits rafts to travel accross intestinal epithelium.

In this study, we have shown that a (glyco)sphingolipid binding domain structurally related to the V-3 loop of the gp120 HIV-1 protein can be detected on BSDL using incremental combinatorial extention method (Shindyalov and Bourne, 1998). This V-3 like domain may be essential to the association of BSDL with raft microdo-

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mains whose integrity is essential to BSDL secretion by pancreatic acinar cells.

Results

Characterization of a V3-like Domain on BSDL

One hallmark of the human immunodeficiency virus (HIV)-1 binding to cell surface during infection is a specific interaction with glycosphingolipids (GalCer, globotriaosylceramide) present in lipid rafts (Guex and Peitsch, 1997; Fantini et al., 2000; Mahfoud et al., 2002b; Hug et al., 2000). This binding is mediated by the V3 loop of the surface envelope glycoprotein gp 120 (Guex and Peitsch, 1997). In solution the V3 loop adopts a typical hairpin fold, which has been also characterized in amyloid proteins (Fantini et al., 2002; Mahfoud et al., 2002a). The possible association of BSDL with rafts in pancreatic microsomes (Bruneau et al., 1995; Bruneau and Lombardo, 1995) led us to investigate whether such a V3-like structural consensus domain is present on BSDL. For this purpose structure similarity searches were performed using the combinatorial extension method (CE) (Shindyalov and Bourne, 1998) and a structural alignment of BSDL and the V3 loop motif of the gp120 protein has be obtained (Figures 1A and 1B). The V3-like domain of BSDL is comprised between residues Asp361 and Leu393. This motif is a hairpin structure consisting of a helix-turn-helix motif formed by the end of helix α_J (amino acids 355–368), followed by the β-turn (amino acids 369–376), and the beginning of helix α_K (amino acids 377–407) of the BSDL structure (Wang et al., 1997). The HIV-1 V3 loop motif contains aromatic residues that mediate binding to individual sugar rings of glycosphingolipids (Fantini et al., 2002; Mahfoud et al., 2002b). Interestingly, there is also an aromatic residue (i.e., Trp371) in the BSDL motif that could be essential for sphingolipid binding. This residue is found in a symetrical position with regard to residues Phe20 or Tyr21 of the gp120 V3 loop (Figure 1B). This loop is exposed to the solvent and fully accessible on the surface of BSDL, consistent with a potential role in sphingolipid binding (Figure 1C). Finally, alignment parameters provided by the CE program suggest that the structural similarity between the gp120 V3 loop and the sequence 361–393 of BSDL is highly significant (Table 1). In particular, the root-mean-square-deviation was <4 Å and the statistical significance of the alignment (Z-score) was >3.

The V3-like Domain of BSDL Is a Sphingolipid Binding Domain

The identification of a V3-like domain on BSDL prompted us to study the interaction of this domain with glycosphingolipids (GalCer and GlcCer) and sphingomyelin. A peptide derived from the bovine BSDL sequence from Asp361 to Leu393 (NATYEVYTEPWAQDSSQTETRKKT MVDLETDIL) was synthesized and its interaction with sphingolipids was analyzed using the Langmuir film balance as previously described (Mahfoud et al., 2002a). In these experiments, the synthetic peptide was added to the aqueous subphase underneath the monomolecular film formed by lipids at the water-air interface, and the resulting interaction was measured as an increase in the surface pressure of the film (Mahfoud et al., 2002a). A preliminary experiment demonstrated that the BSDL synthetic peptide did not affect the surface tension of water in absence of lipid (data not shown). In contrast, this peptide interacted specifically with GalCer and the interaction was dose dependent (Figure 2A). The maximal increase in surface pressure ($\Delta\pi$ max $=$ 20 mM/m) was obtained for a peptide concentration of 400 nM, with a half-maximal effect at 50 nM. Overall, data obtained with the BSDL synthetic peptide were consistent with those previously obtained with synthetic peptides derived from the gp120 V3 loop, the Alzheimer β-amyloid peptide, and the cellular prion protein (Mahfoud et al., 2002a).

To assess the specificity of the interaction with sphingolipids of the BSDL synthetic peptide, monomolecular films of GalCer were prepared at various initial pressures (πi), and the maximal increase in surface pressure ($\Delta\pi$ max) induced by the synthetic peptide on these films was determined after equilibrium has been reached. As shown in Figure 2B, $\Delta\pi$ max gradually decreased whereas πi increased. The critical pressure of insertion (i.e., the theoretical value of πi extrapolated for $\Delta\pi$ max $=$ 0 mN/m) was 45 mN/m. This value is greater than the mean density of lipids in cell membranes, which has been estimated around 30 mN/m (Larson and Quinn, 1994). A specific interaction was also observed with GlcCer. In this case, below 25 mN/m, a πi value corresponding to a fluid disordered (Lc) phase, the $\Delta\pi$ max induced by the BSDL synthetic peptide, was between 5 and 10 mN/m (Figure 2C). At a πi of 25 mN/m, the value of $\Delta\pi$ max reached 20 mN/m, then for values of πi above 25 mN/m, a pressure which does correspond to liquid-ordered (Lo) phase domains, $\Delta\pi$ max gradually decreased as πi increased and reached a critical pressure of insertion at approximately 46 mN/m. From these data, one could conclude that the interaction of the BSDL synthetic peptide with GalCer and GlcCer is optimal when glycosphingolipids form a densely packed monolayer, a molecular organization likely preponderant in the liquid-ordered phase of lipid rafts (Brown, 1998).

Contrary to previously characterized V3-like domains (Mahfoud et al., 2002a), the synthetic peptide of BSDL did not bind specifically to sphingomyelin (Figure 2B). Indeed, the peptide did not increase the pressure of a sphingomyelin film above a πi of 25 mN/m. This result is not surprising if one considers that in the Glu200 Lys mutant of the prion protein, the resulting redistribution of surface charges dramatically affected binding to sphingomyelin but had no effect on GalCer recognition (Mahfoud et al., 2002a). Examining the V3-like loop derived from the BSDL sequence suggests that its structure in aqueous solution could be stabilized by saline bridges between Arg380 and Asp374, and between Lys382 and Glu377 (Figure 3). Accordingly, these charged residues are no more susceptible to interact with the charged polar head group of sphingomyelin.

BSDL Cofractionates with Caveolin-1 of Raft Microdomains

It is now clearly established that (glyco)sphingolipids are essential constituents for the formation of rafts (Si-

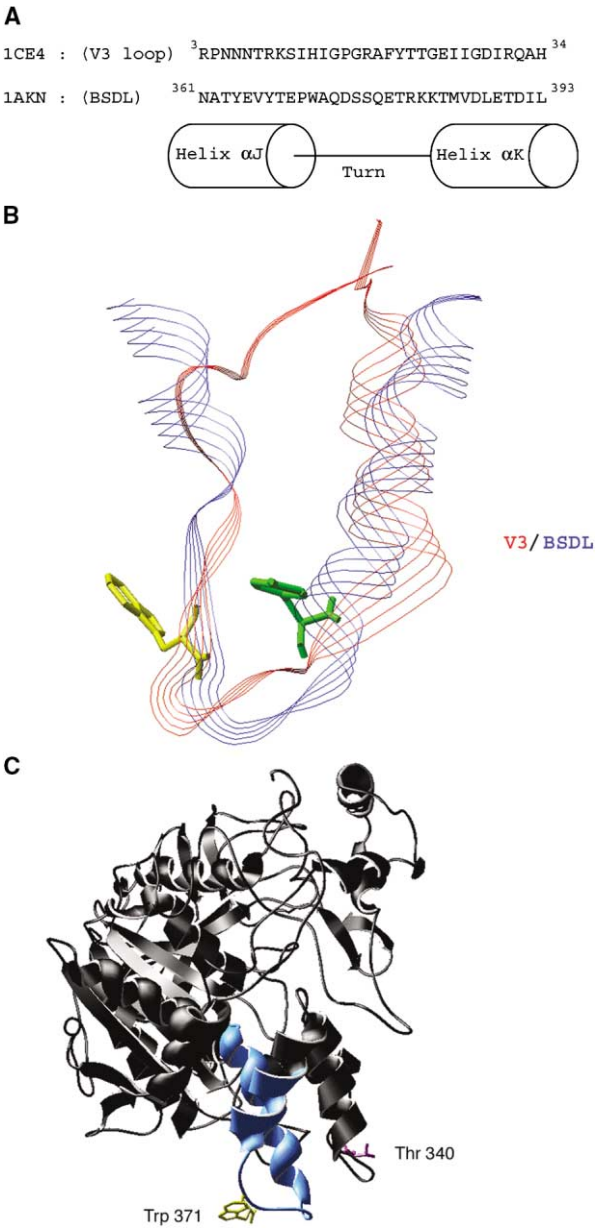

Figure 1. Structural Homology between HIV-I gp120 and BSDL

(A) Structure-based sequence alignment of HIV-I gp120 (V3 loop) and BSDL (sequence 361–393). For each sequence, PDB entry name as well as starting and ending residues are given. Alignment parameters are given in Table 1.

(B) Superimposition of the sphingolipid binding motif in the V3 loop of HIV-I gp120 (red) and BSDL (blue). The lateral chains of the aromatic residues potentially involved in binding to glycosphingolipid are shown; BSDL Trp371 in yellow, V3 loop Tyr21 in green.

(C) Location of the V3-like domain (blue) in the three-dimensional structure of BSDL. Residues Trp371 (yellow) and the phosphorylation site Thr340 (purple) are indicated.

mons and Ikonen, 1997; Brown and London, 2000); therefore, we have investigated whether BSDL can associate with lipids rafts as suggested by the presence of the V3-like domain on BSDL and by previous data (Bruneau et al., 1995; Bruneau and Lombardo, 1995). For this purpose, we have isolated raft microdomains from the human pancreatic SOJ-6 cells using a step sucrose gradient. Gradient fractions were examined by SDS-PAGE and Western blot using antibodies to caveolin-1 to locate raft microdomains (Schubert et al., 2002) and pAbL64 to detect the human BSDL. As shown in Figure 4A, caveolin-1 colocates with BSDL within fractions 2–5, whereas Rab5, a cytosolic protein associated with endosomes trafficking is mainly associated with soluble fractions 8 and 9. Of course, BSDL is also present in soluble fractions as the result of detergent lysis of secretory vesicles where mature soluble BSDL accumulates (Bruneau and Lombardo, 1995). We also used the CHO-K1 cells transfected with the cDNA of the rat BSDL referred to as the CHO-3B clone (Bruneau et al., 1997). Rat BSDL which locates with Rab5 within solubles fractions 8 and 9, can also be detected in fractions 2–5 which are enriched in caveolin-1 (Figure 4B). Consequently, in CHO-3B cells expressing and secreting recombinant rat BSDL (Bruneau et al., 1997), as well as in pancreatic SOJ-6 cells which constitutively expressed human BSDL (Pasqualini et al., 1998), a fraction of the enzyme cofractionates with caveolin-1 and likely colocates with raft microdomains.

Another possibility is that secreted BSDL, by means of the V3-like domain, can be recaptured after its association with rafts microdomains of the acinar cell plasma membrane. To answer this specific point, we have performed a biotinylation of SOJ-6 cell surface proteins.

Table 1. Structure-Based Alignment Parameters Obtained with the CE Program

CE Parameter[a]	1AKN versus 1CE4
Rmsd (Å)[b]	3.7
Z-score[c]	3.1
Sequence identity (%)	0
Aligned/gap positions[d]	32/0

The proteins are identified with their PDB entry numbers: 1CE4, HIV-I gp120 V3 loop; 1AKN, BSDL.

[a] Provided by the CE program using the two chains alignment routine (the resulting alignments are shown in Figure 1). Structure similarity searches were performed with default parameter, except for the "select similarity level" option ("high" instead of "medium" in order to obtain a close match).

[b] Root-mean-square deviation, rmsd (should be <5 Å since members of the same protein family that obviously have the same fold can differ by up to 4 Å or more in rmsd).

[c] Measure of statistical significance of the result relative to an alignment of random structures (range 2.5–5.0). The Z-score is generally used to filter less significant results or alternatively look for weak similarities. The relatively low values of the Z-score (i.e., <3.5) may be explained by the absence of sequence identity between the aligned motifs.

[d] Ideally, the number of gap positions in alignment between two chains should be less than 20% of aligned residue positions.

For this purpose, SOJ-6 cells were grown until confluence, then cell surface proteins were biotinylated, and cells were washed and lysed. The material was loaded on streptavidin-agarose beads and the biotinylated material eluted by acetic acid was further separated on SDS-PAGE and electrotransferred onto nitrocellulose membranes. The membranes were finally probed with antibodies to biotin and to BSDL (Figure 5). Results showed that many proteins were reactive with antibodies to biotin (lane 1, 0.5 μg of biotinylated proteins); however, except for a 32 kDa peptide, no material associated with the size of human BSDL (i.e., ~120 kDa) was probed with pAbL64 (lane 2, 5 μg of biotinylated proteins). For comparison, lane 3 displays the migration of mature BSDL from SOJ-6 cell culture medium. This result suggests that BSDL does not associate with rafts at the level of the cell plasma membrane. Therefore BSDL associates with intracellular raft microdomains, and this binding likely involves the V3-like domain of the enzyme and the (glyco)sphingolipids of rafts.

Disorganization of Membrane Raft Structure Decreases the Secretion Rate of BSDL

Since the sequence of the BSDL synthetic peptide corresponds to a loop located at the surface of the enzyme (Figure 1C) and since pancreatic BSDL associates with rafts (Figure 4), we wondered what could be the physiological relevance of this glycosphingolipid binding domain in BSDL secretion. Pharmacological manipulation of membrane lipid levels with well-documented drugs has been used to address the role of lipid rafts in many systems. For this purpose we have used methyl-β-cyclodextrin a drug described to deplete cholesterol in membrane rafts (Chen and Resh, 2002). At drug concentrations devoid of cytotoxicity, methyl-β-cyclodextrin had no effect on BSDL secretion by CHO-3B cells (Figure 6A). In contrast, the sequestration of cholesterol

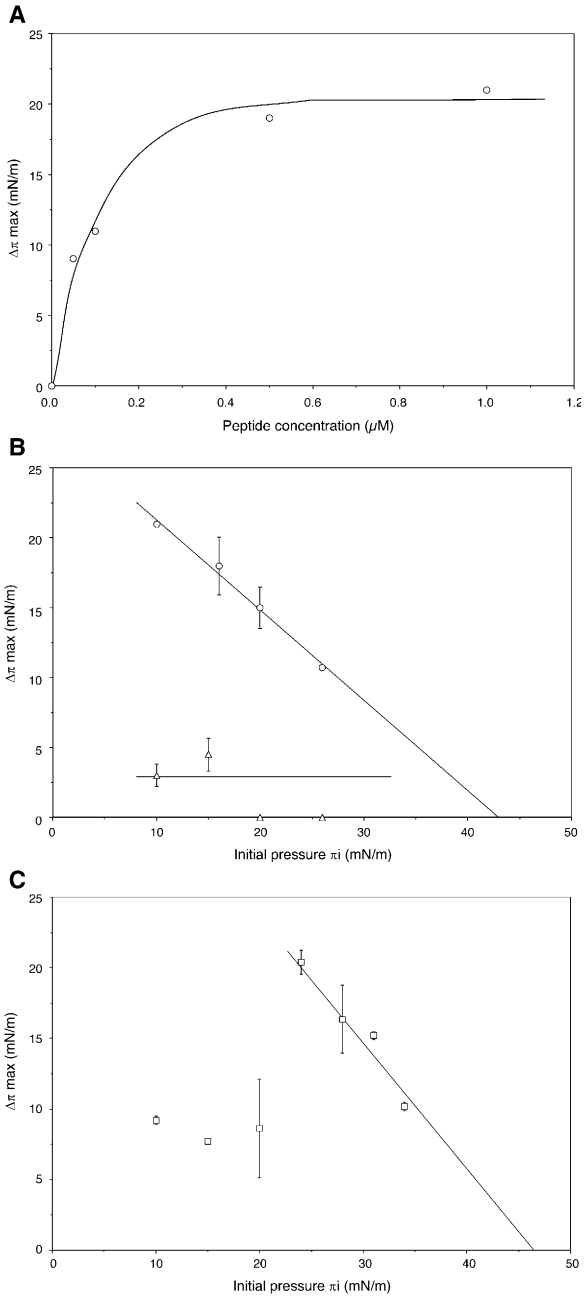

Figure 2. Binding of the BSDL-Derived Synthetic Peptide to Monomolecular Film of Glycosphingolipids

(A) Dose-dependent interaction between the BSDL-derived synthetic peptide and monomolecular film of GalCer prepared at an initial pressure (πi) of 10 mN/m. Increases in the surface pressure induced by the synthetic peptide added to the aqueous subphase at the concentration as indicated, were determined at equilibrium. (B and C) Maximal surface increase (Δπ max) reached after injection of BSDL synthetic peptide under GalCer (circles) and sphingomyelin (triangles) (Figure 2B) or GlcCer (squares) (Figure 2C) films at various initial surface pressures (πi). The synthetic peptide was used at a final concentration of 0.5 μM. Results are means ± SD of three independent experiments and invisible error bars are within symbol size.

A

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HUMBSDL     KTTFDVYTESWAQDPSQENKKKTVVDFETDVL
FERBSDL     KTTFDVYTESWAQDPSQENKKKTVVDFETDVL
RATBSDL     QATFDIYTESWAQDPSQENMKKTVVAFETDIL
MOUBSDL     QATFDIYTESWAQDPSQENMKKTVVAFETDVL
BOVBSDL     NATYEVYTEPWAQDSSQETRKKTMVDLETDIL
            :.*.::***.****.***:.***:**:***:*
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B

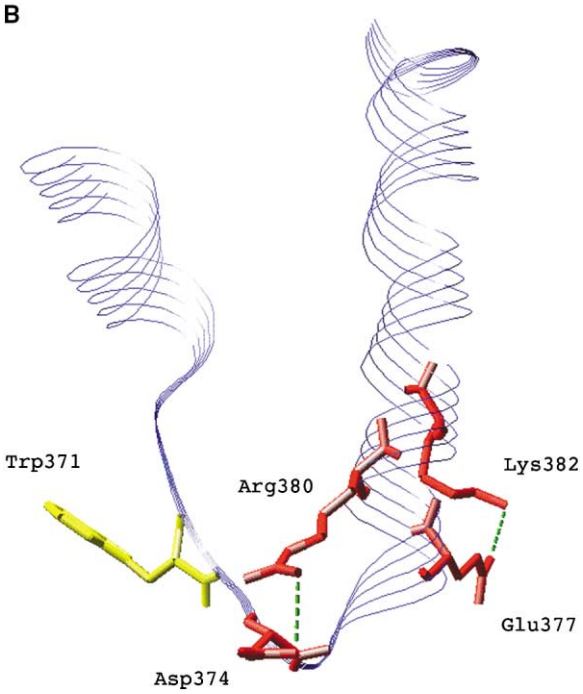


Figure 3. Modeling of the Synthetic Peptide Structure Derived from the Bovine BSDL

(A) Sequence alignments showing that many charged residues are conserved in bovine (BOVBSDL), human (HUMBSDL), ferret (FERBSDL), mouse (MOUBSDL), and rat (RATBSDL) corresponding sequences (Sbarra et al., 1998). Asterisks indicate matches, double dots indicate conserved residues, and single dots indicate mismatches.

(B) The model based on the three-dimensional structure of BSDL shows potential saline interactions (between Arg380 and Asp374, and between Lys382 and Glu378) in the BSDL synthetic peptide.

(Chen and Resh, 2002) by filipin (Figure 6B) induced a dramatic decrease in the secretion rate of BSDL.

Sphingolipids also participates in raft structure; therefore, we used metabolic inhibitors of (glyco)sphingolipid biosynthesis. In the first instance we have used inhibitors acting early in sphingolipid synthesis such as L-cycloserine (an inhibitor of serine palmitoyltransferase) and Fumonisin 1 or 2 (both inhibitors of dihydroceramide synthetase). Although used at efficace concentration (10 and 20 μM for L-cycloserine and Fumonisin, respectively) none of these inhibitors interferes with the secretion of BSDL by CHO-3B cells (data not shown). We therefore turned to inhibitors acting later in sphingolipid synthesis such as D609 (partly an inhibitor of sphingomyelin synthesis) and PDMP (an inhibitor of glycosphingolipid synthesis) to alter membrane rafts composition and to disturb their structure (Lefrancois et al., 2002).

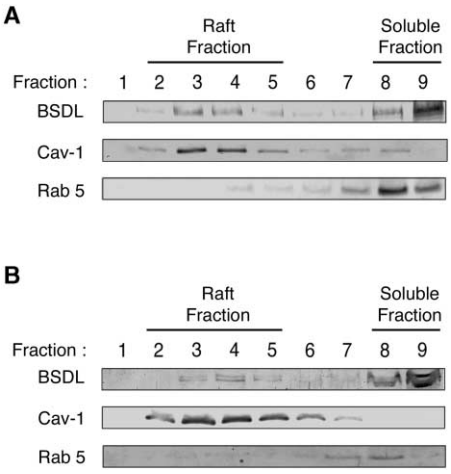


Figure 4. Localization of BSDL in Caveolin-1-Rich Membrane Fraction

(A) SOJ-6 human pancreatic cells were treated with Brij98 to isolate raft microdomains which were monitored by the distribution of caveolin-1 over the step sucrose gradient. Fractions of the sucrose gradient (30 μl) were analyzed by SDS-PAGE and electrotransferred onto nitrocellulose membranes. These membranes were probed with antibodies to caveolin-1 (Cav-1), with pAbL64 against human BSDL (BSDL), or with antibodies to Rab5 (Rab5).

(B) CHO cells expressing the rat BSDL (CHO-3B clone) were treated and analyzed as in (A), excepted that the rat BSDL was probed with the pAbL10.

As shown in Figure 7A, D609 totally inhibited the secretion of BSDL by CHO-3B cells. Because D609 also partially inhibited the BSDL activity, we have checked by Western blot using pAbL10 that the protein is actually absent from the cell culture medium upon treatment of CHO-3B cells with the drug. As shown in Figure 7B in the absence of D609, BSDL can be detected in the cell culture medium after 4 hr incubation, and then the secreted amount of BSDL increases with time, whereas no BSDL protein can be detected in the culture medium of D609-treated CHO-3B cells. In addition, the depletion of glycosphingolipids in rafts of CHO-3B cells with PDMP significantly slowed the secretion rate of BSDL (Figure 7C). This result suggests that BSDL exploits (glyco) sphingolipids of lipid rafts during its secretion process.

Cells in culture are estimated to internalize via endocytosis about half their plasma membrane per hour (Steinman et al., 1983). This event is followed by a parallel process of recycling, remodeling, and resynthesis of membrane components, in part lipids, in order to maintain the cell surface composition as dynamically constant. Therefore, the fact that early inhibitors of sphingolipid synthesis are uneffective in decreasing the secretion rate of BSDL suggests that the salvage pathway recycling surface glycosphingolipids (Tettamanti et al., 2003) is sufficient to insure the formation of lipid rafts in CHO-3B cells and able to drive BSDL toward secretion. This salvage pathway in part connects lysosomes to the Golgi apparatus (Chen et al., 1998); consequently, the lysosomal hydrolase inhibitor chloroquine causes the arrest of the metabolic salvage pathway (Tettamanti et al., 2003). As shown in Figure 8, incubation of CHO-3B cells with chloroquine (1 mg/ml) caused a

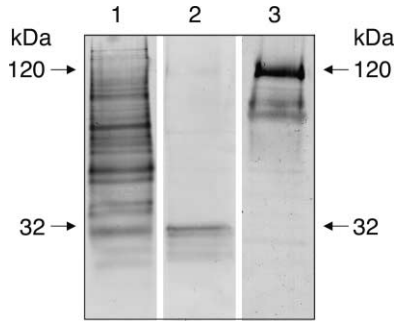


Figure 5. Immunodetection of Biotinylated Cell Surface Proteins in SOJ-6 Cells Lysate

SOJ-6 cells were grown until confluence, and membrane proteins were biotinylated, and finally cells were lysed. Then biotinylated membrane proteins were purified on a streptavidin-agarose affinity column. The material eluted with 0.1 M acetic acid was separated on SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed with anti-biotin antibodies (lane 1, 0.5 μg of biotinylated proteins) and pAbL64 (lane 2, 5 μg of biotinylated proteins). Lane 3 displays an immunodetection experiment using pAbL64 on secreted material (i.e., material associated with the SOJ-6 cells culture medium). Arrow indicates the apparent molecular mass of detected proteins.

significant inhibition of BSDL secretion. These data suggested that (i) the salvage pathway recycling membrane lipids between lysosomes and the Golgi apparatus is active in CHO-3B cells, (ii) this pathway is large enough to assume the formation of lipid rafts in these cells and the resulting secretion of BSDL, and (iii) BSDL associates with lipid rafts in the Golgi apparatus.

Discussion

In the present study, we have identified a specific sphingolipid binding domain in BSDL. This peptide domain has sequence identity neither with the HIV-V3 loop peptide nor with the prion peptide or with the Alzheimer β-amyloid peptide (Mahfoud et al., 2002a). However, all these peptides present a similar structure consisting of a helix-turn-helix motif. For BSDL, this motif is constituted by the helix α_J and helix α_K and the domain in between. Crystallography studies showed that this domain is located at the surface of the protein (Wang et al., 1997). We have also demonstrated that the V3-like motif of BSDL interacted with reconstituted raft-like monolayers of GalCer and GlcCer, but not with sphingomyelin. In the latter case, it can be reasonably argued that electrostatic interactions between basic and acid residues could stabilize the structure of the synthetic BSDL peptide in solution and consequently impair its binding to the polar head group of sphingomyelin. However, in native BSDL the charged amino acids of the V3-like domain may be bridged to residues located elsewhere in the BSDL structure. Alternatively, one cannot exclude that residues outside the V3-like domain, characterized in the present study, could mediate the binding of BSDL to sphingomyelin.

The structure and the binding of the synthetic peptide derived from BSDL to glycosphingolipids could be fortuitous and without physiological relevance. Therefore, we

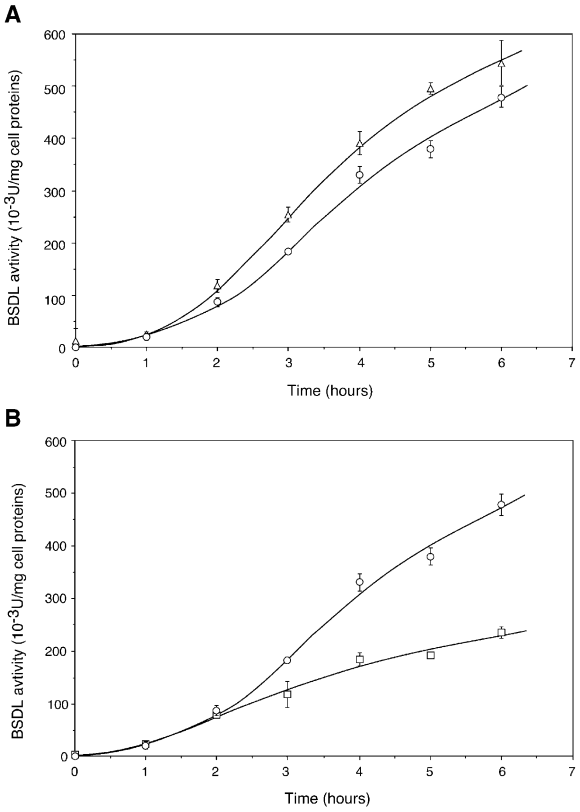


Figure 6. Effect of Drugs Affecting Cholesterol on BSDL Secretion

CHO-3B cells secreting the rat BSDL were cultured in Ham's F12 medium until 80% confluence. At time zero the culture medium was replaced by fresh medium containing methyl-β-cyclodextrin (20 mM, [A]) or filipin (1 μM, [B]). Aliquots of the culture medium were collected after each time period of incubation as indicated and the BSDL activity was recorded. Results are means ± SD of three determinations. The cytotoxicity of these drugs at each incubation time period was <10% that induced by Triton X-100 (2% in cell culture medium). Triangles, methyl-β-cyclodextrin; squares, filipin; circles, control.

continued this study and showed that BSDL cofractionates with caveolin-1, taken as raft domain marker, in a step sucrose gradient following lysis of the human pancreatic SOJ-6 cells expressing an oncofetal variant of BSDL (Pasqualini et al., 1998) and of the CHO-3B cells, obtained after transfection of CHO-K1 cells with the cDNA of rat BSDL (Bruneau et al., 1997). These data suggested that BSDL associates with lipid rafts in these two cell models. This result is substantiated by the presence of a fraction of BSDL in the detergent-insoluble phase fraction of human (Bruneau et al., 1995) and rat (Bruneau and Lombardo, 1995) pancreatic cell microsomes or in rat pancreatic zymogen granules (Withiam-Leitch et al., 1995) which contain GPI-anchored proteins associated with lipid rafts (Hooper and Bashir, 1991). Sequence analyses suggested that this domain might be present on BSDL independently of species origin. We continue this study by determining the effect of drugs described to affect raft integrity on BSDL secretion. Because SOJ-6 cells expressed (Pasqualini et al. 1998) but poorly secreted BSDL (Caillol et al., 2000), we used the

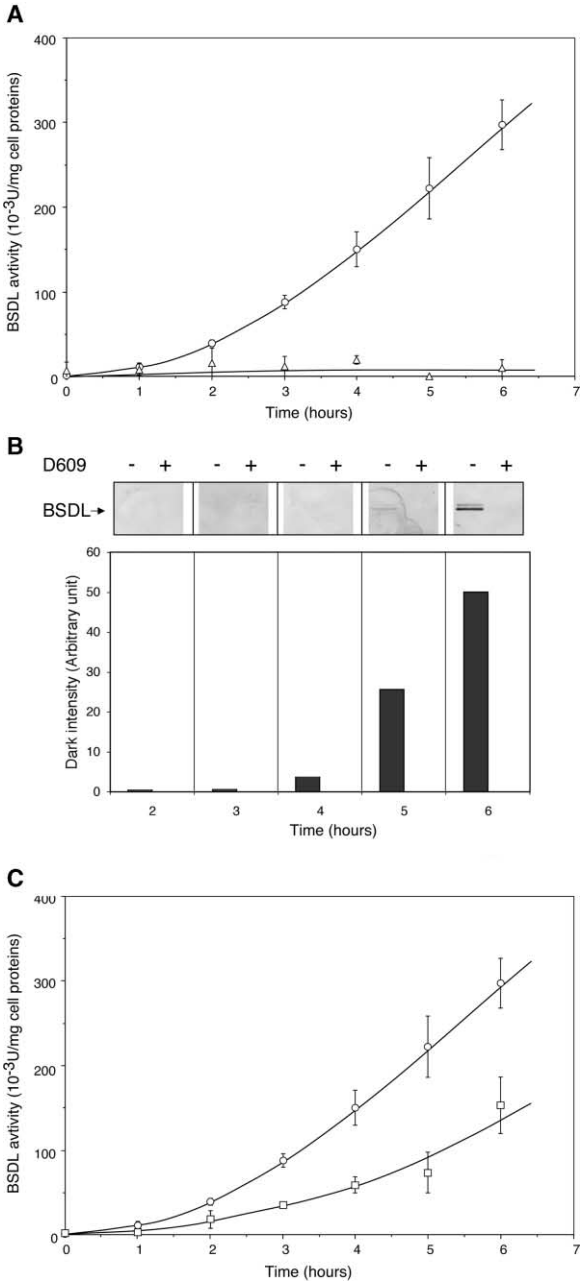


Figure 7. Effect of Drugs Affecting Sphingolipids on BSDL Secretion

CHO-3B cells were cultured as described above in the legend to Figure 6. At time zero of incubation, the cell culture medium was replaced with fresh medium containing D609 (375 μM, [A]) or PDMP (60 μM, [C]), aliquots of the culture medium were withdrawn after the time period of incubation as indicated, and the BSDL activity was recorded. Results are means ± SD of three determinations. The cytotoxicity of these drugs at each incubation time period never reached 15% of that promoted by Triton X-100 (2%) in cell culture experiments (triangles, D609; squares, PDMP; circles, control). Figure 7B displays a typical immunodetection experiment performed on cell culture medium samples (50 μl) taken at the time indicated, of CHO-3B cells either treated (+) or untreated (−) with D609 (375 μM). Quantification of Western blots was performed using the NIH-Image program at http://rsb.info.nih.gov/nih-image/.

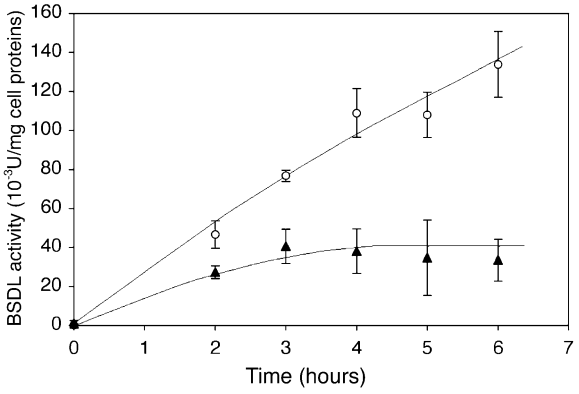


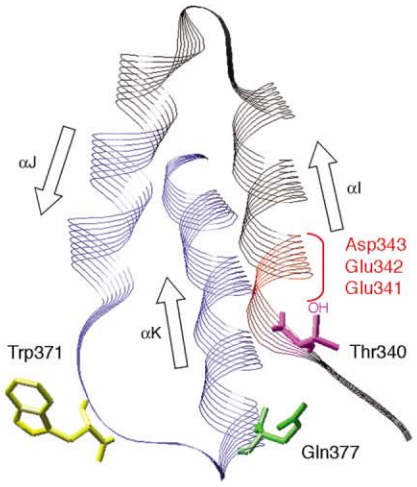
Figure 8. Effect of Chloroquine on BSDL Secretion

CHO-3B cells were cultured to confluence. At time zero of incubation the cell culture medium was replaced with fresh medium containing chloroquine (1 mg/ml, closed triangles), and aliquots of the culture medium were withdrawn after time periods of incubation as indicated, and finally the BSDL activity was recorded. A control experiment was performed in the absence of chloroquine (circles). The cytotoxicity of this drug at each incubation time period never reached 15% of the Triton X-100 (2%)-treated cells. Each value is mean ± SD from four independent experiments.

CHO-3B clone as a secretory cell model of rat BSDL. First, we showed that cholesterol sequestration by filipin impaired BSDL secretion, whereas cholesterol depletion by methyl-β-cyclodextrin had no effect at concentrations below cytotoxicity. It may be difficult to conceive that the way of cholesterol removal, e.g., depletion versus sequestration, could differently affect BSDL secretion. A simple explanation to this apparent discrepancy is that methyl-β-cyclodextrin is impermeable (Awasthi-Kalia et al., 2001) and is only able to affect the structure of raft microdomains located at the plasma membrane of cells, and we have seen that BSDL may not be associated with cell surface rafts. Consequently, and as already observed with the surface sorting of the GPI-anchored placental alkaline phosphatase (Lipardi et al., 2000), cholesterol removal from the cell surface did not affect the exocytosis of BSDL. Another way to disrupt lipid rafts was to inhibit the biosynthesis of (glyco)sphingolipids, which are major components of microdomains, with D609 or PDMP. As expected, these inhibitors decreased the secretion rate of BSDL, confirming that the disorganization of lipid rafts largely affected the secretion process of BSDL. Therefore, one has to conclude that the V3-like loop of BSDL is a sphingolipid binding domain, or SBD (Fantini, 2003), that is involved in BSDL binding to rafts and in secretion.

The question now is when does the BSDL SBD become functional and bind to lipid rafts. An informative point that could be linked to the CHO-3B cell model used in this study is that chloroquine, a lysosome hydrolase inhibitor, decreases the secretion rate of BSDL, whereas loading cells with GlcCer or sphingomyelin enhances the enzyme secretion (data not shown). This suggests that the salvage processes of membrane lipids controlling the active concentration of ceramides and sphingolipids is important enough in CHO-3B cells to assume the formation of raft microdomains that BSDL associ-

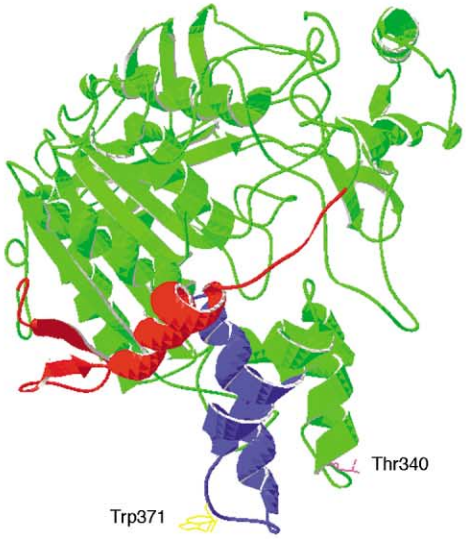
A



B

```
HUMBSDL     TEEDFYKLVS
FERBSDL     TEEDFYKLVS
RATBSDL     TEEDFYRLVS
MOUBSDL     TEEDFYRLVS
BOVBSDL     TEEDFYKLVS
            ******:***
```

C



D

```
HUMBSDL PTHWEPYTTENSGYLEITKKMGSSSMKRSLRTNFLRYWTLTYLAL
FERBSDL PTHWDPYSIESGNYLEITKKMDGHSMKQHLRASYLRYWTLTYEAL
RATBSDL PTHWYPYTMENGNYLDINKKITSTSMKEHLREKFLKFWAVTFEML
MOUBSDL PTHWYPYTLENGNYLDITKTITSASMKEHLREKFLKFWAVTFEVL
BOVBSDL PANWDPYTLEDDNYLEINKQMDSNSMKLHLRTNYLQFWTQTYQAL
        *..*.**:.*:..**:*.*.....***..**..:*:.*..*..**
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Figure 9. Hypothesis on Threonine 340 Phosphorylation and BSDL Release from Rafts

(A) The ribbon model shows that the phosphorylatable Thr340 residue of helix α_I is proximal to the Gln377 residue of the V3-like loop of BSDL and the helix α_I. The phosphorylation of Thr340 could result in structural modifications of the helix α_I via electrostatic repulsion of negatively charged Glu341, Glu342, and Asp343 residues and attraction of the helix α_K Gln377 residue. Finally, this could lead to the release of BSDL from membranes and secretion.

(B) The sequence following the Thr340 residue is perfectly conserved in bovine (BOVBSDL), human (HUMBSDL), ferret (FERBSDL), mouse (MOUBSDL), and rat (RATBSDL) corresponding sequences (Sbarra et al., 1998). Asterisks indicate matches and double dots conserved residues.

(C) The ribbon model displays the sequence 490–534 (red) involved in the proper folding of BSDL (DiPersio et al., 1994). The V3-like loop is in blue.

(D) Sequence homologies between residues 490 and 534 of BSDL (see [B] for legend).

ates with. This salvage pathway that covers up to 90% of total glycosphingolipid metabolism in human fibroblast (Tettamanti et al., 2003) connects lysosomes to the Golgi apparatus. Furthermore, in SOJ-6 cells the raft-associated fraction of BSDL was reactive with the mAbJ28 (data not shown) which recognizes terminal fucose linked to glycans of the protein in the *trans*-Golgi compartment (Panicot et al., 1999; Colley, 1997). We have already shown that BSDL is released from intracellular membranes once *N*- and *O*-glycosylation were completed (Abouakil et al., 1993; Bruneau et al., 1997) in a genistein-sensitive compartment, likely the *trans*-Golgi network (Pasqualini et al., 2000), upon phosphorylation of residue Thr340 by a casein kinase II (Vérine et al., 2001; Pasqualini et al., 2000). Then, BSDL is mainly associated with the soluble protein fraction in zymogen granules (Bruneau et al., 1995). Therefore, phosphorylation on Thr340 may be the *trans*-Golgi network event that induces BSDL release from lipid rafts. Taken as one, these results indicate that BSDL is transiently associated with rafts from the Golgi compartment up to the *trans*-Golgi network.

From this point, another important issue is to explain how BSDL is released from lipid rafts to be secreted. Thr 340 residue locates at the end of the helix α_I which is parallel to helix α_K (Wang et al., 1997). As a consequence, Thr340 is in a vis-à-vis position of a polar residue Gln377 of the BSDL SBD (Figures 9A and 9B). Therefore the bulky and negatively charged phosphoryl group of the phosphorylated Thr 340 could perturbate the helix α_I structure via a repulsive effect on negatively charged Glu341, Glu342, and Asp343 residues and via an attractive effect on positive Gln377 residue of the helix α_K. This may generate a conformational change in the V3-like loop structure that in turn may lead to dissociation. Clearly the simple switch of charge at position Glu200 into Lys200 profoundly modifies the binding to sphingolipids of the V3-like peptide derived from the mutant of the prion protein associated with the Creutzfeldt-Jakob disease (Mahfoud et al., 2002a). The possible conformational change occurring during the release process is enlighted by the lack in activity of the membrane-associated BSDL that gains full activity once released from membranes (Bruneau et al., 1995; Bruneau and Lombardo, 1995). Interestingly, it has been shown that the sequence 490–534 of BSDL is required for normal intracellular processing and secretion of the enzyme (DiPersio et al., 1994). The ribbon model (Figure 9C) indicates that this domain is close to the BSDL SBD. Once deleted, this sequence 490–534 (which presents homologies between species, Figure 9D) induced the retention of the unproperly folded and inactive enzyme in membrane microsome fraction (DiPersio et al., 1994). Therefore, unfolded BSDL is associated with the membrane, and it has been shown that Grp94 is involved in this associa-

tion (Bruneau et al., 1997). All these results suggest that Grp94 achieves the folding of BSDL, and that only properly folded BSDL may be completely glycosylated (Bruneau and Lombardo, 1995), in part *O*-glycosylated at the level of the C-terminal domain of BSDL (Wang et al., 1995). Only BSDL molecules that have completed these steps are able to associate with lipid rafts. Thus, the correct folding of the SBD of BSDL may determine the right conformation of the whole BSDL molecule. Therefore, only correctly folded BSDL molecules could be susceptible to bind to rafts (or related structures) and to become accessible to phosphorylation on Thr340 by a casein kinase (Pasqualini et al., 1997) and further secreted.

In conclusion, our study underscores the capacity of the incremental combinatorial extension method developed by Shindyalov and Bourne (1998) to identify structurally related domains sharing common functional properties in proteins without sequence identity (Mahfoud et al., 2002a). In this respect the characterization of a structurally conserved sphingolipid binding domain potentially involved in the binding to lipid raft components may be a typical example of such functional identity that is independent of sequence homology (Fantini, 2003). A functional sphingolipid binding domain structurally related to the V3 loop of HIV-1 gp120 has already been delineated in prion and Alzheimer proteins (Mahfoud et al., 2002a). Results of the present study further show that a similar domain can be found in cellular proteins such as BSDL, which associates with rafts during secretory processes. From these data, one can anticipate that the presence of a surface-exposed SBD as detected by the incremental CE program on proteins of known structure may support binding to lipid rafts (or to related structures).

Experimental Procedures

Materials

The chemicals used in this study including GlcCer, GalCer, sphingomyelin, PDMP, methyl β-cyclodextrin, filipin, Brij98, Triton X-100, D609, and alkaline phosphatase-labeled antibodies to rabbit and mouse immunoglobulins were from Sigma. The synthetic peptide derived from the bovine BSDL sequence was purchased from Q-Biogene (Evry, France). The peptide was purified by high-performance liquid chromatography (purity >95%) and characterized by mass spectrometry (experimental *Mr* of 3761.99, theoretical *Mr* of 3763.1). Polyclonal antibodies to human BSDL (pAbL64) and to rat BSDL (pAbL10) were homemade (Bruneau et al., 1995; Aubert et al., 2002), and polyclonal antibodies to caveolin-1 were from Santa Cruz Biotechnology (Santa Cruz, CA).

Structure Analysis

Structure similarity analyses were performed using the two chains calculation routine of the CE combinatorial extension program (Shindyalov and Bourne, 1998), as previously described (Mahfoud et al., 2002a). Briefly, the CE program aligns two polypeptide chains using characteristics of their local geometry as defined by vectors between carbon α positions. Molecular structures were visualized using the Swiss-PDB viewer (Guex and Peitsch, 1997). PDB identification numbers were 1CE4 for HIV-1 gp120 and 1AKN for BSDL, respectively.

Surface Pressure Measurements

The surface pressure was measured with a fully automated microtensiometer (μTROUGH SX-Kibron Inc.) as already described (Mahfoud et al., 2002a). Pressure increases were recorded at 20°C until the equilibrium was reached (maximal surface pressure increase $\Delta\pi$ max) as indicated elsewhere (Hammache et al., 1998). The data were analyzed with the Filmware 2.3 program (Kibron Inc.).

SOJ-6 Cell Culture

Human pancreatic SOJ-6 cells expressing an oncofetal variant of BSDL were kindly provided by Dr. M.-J. Escribano (INSERM, Marseille) and cultured as previously described (Pasqualini et al., 1998).

CHO-K1 Cell Culture and Incubation with Drugs

CHO-K1 cells transfected with the full-length cDNA encoding the rat BSDL and referred to as the CHO-3B clone were cultured as already described (Bruneau et al., 1997). Cells were grown in 3 cm diameter Petri plates until 80% confluence was reached, then at time zero of incubation the cell culture medium was replaced by fresh medium containing effectors at a final concentration as mentioned. Control experiments were performed in the absence of drug.

Biotinylation and Streptavidin Precipitation of Cell Surface Proteins

SOJ-6 cells were washed three times with PBS; they were then released from culture plates by treatment with a nonenzymatic cell-dissociation solution (Sigma) for 15 min at 37°C. The cells were suspended at a concentration of 25×10^6 cells/ml in ice-cold PBS (pH 8.0) and 0.5 mg of sulfo-NHS-LC biotin (PerbioScience, Helsiborg, Sweden) was added per milliliter of reaction volume including Complete EDTA-free mix (Roche Diagnostic) to avoid degradation of proteins. The mixture was incubated at 4°C for 2–3 hr. The cells were washed then three times with ice-cold PBS (pH 8.0) to remove the remaining biotinylation reagent. After centrifugation, the cell pellet was resuspended in an adequate volume of ice-cold PBS, sonicated 15 s twice, and cleared by centrifugation (30 min. 4°C, 13,000 × g). The supernatant, containing biotinylated cell surface proteins (300 μg), was loaded on streptavidin-agarose beads (150 μl, Sigma) and incubated overnight at 4°C under agitation. The beads were exhaustively washed with PBS; then complexes formed of biotinylated proteins bound to streptavidin-agarose beads were dissociated by 0.1 M acetic acid (pH 2.0) for 30 min at 37°C, and centrifuged to collect the supernatant where locate biotinylated proteins. Biotinylated material was separated on SDS-PAGE, electrotransferred onto nitrocellulose membranes, and probed with adequate antibodies.

Enzyme Assays and Protein Determination

BSDL activity was recorded using 4-nitrophenyl hexanoate (Gjellesvik et al., 1994). Except for D609 that decreased by some 30%–40% the BSDL activity, drugs used under the conditions of this study did affect the enzyme activity or were cytotoxic. Protein concentrations were determined using the microBCA kit (Pierce).

Raft Isolation

Raft microdomains were isolated according to Drevot et al. (2002). Briefly, SOJ-6 or CHO-3B cells (2×10^8) were suspended in 1 ml of buffer A (25 mM HEPES, 150 mM NaCl, 1 mM EGTA, 5 mM Na_3VO_4, 10 mM NaPP, 10 mM NaF, and protease inhibitors) and homogenized by sonication (5 × 5 s, 4 w, 4°C, Branson sonifier). The postnuclear supernatant obtained by centrifugation of the homogenate (1260 × g, 10 min, 4°C) was then incubated at 37°C (to avoid unspecific binding to rafts) in the presence of Brij 98 (1% final concentration). After 5 min solubilization, the homogenate was diluted with 2 ml of buffer A containing 2 M sucrose (final sucrose concentration 1.33 M; final Brij 98 concentration 0.33%) and chilled down on ice (55 min) before being placed at the bottom of a step sucrose gradient (0.9-0.8-0.75-0.7-0.6-0.5-0.4-0.2 M sucrose, 1 ml each) in buffer A. The gradient was centrifuged at 38,000 rpm for 21 hr using a SW41 rotor (Beckman Instruments Inc.) at 4°C. One milliliter fractions were collected from top to bottom, except for the last fraction (fraction Nb. 9), which contains three milliliter. Fractions were further resuspended in the Laemmli's loading buffer (1970).

SDS-PAGE and Western Blot

SDS-PAGE was performed on 7.5% polyacrylamide and 0.1% SDS slab gels as described by Laemmli (1970) using a BioRad Mini Pro-

tean II apparatus (Richmond, WI). After electrophoretic migration, proteins were electrotransferred onto nitrocellulose membranes at 4 mA/cm^2 for 18 hr. The efficiency of electrotransfer was checked by staining the nitrocellulose membrane with 2% Ponceau S solution. Nitrocellulose membranes were air-dried, and the transferred proteins were immunodetected using adequate primary and secondary antibodies. Membranes were developed for 10 min with a mixture of nitro-blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate (0.5 mM each) in 0.1 M Tris/HCl buffer (pH 9.5), 100 mM NaCl, and 1 mM $MgCl_2$.

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